A PHARMACEUTICAL COMPOSITION COMPRISING A RETRO-IVERSO ISOMER PEPTIDE

Technical Field

The present invention relates to therapeutic peptides that possess inhibitory activity towards elevation of serum triglyceride (triacylglycerol) levels, an unavoidable result that occurs upon ingestion of meals containing high composition of fat. Administration of such peptides before or concomitantly with meals allows for less net absorption of fatty acids into the system, thereby contributing to the prevention of various known cardiovascular diseases as well as obesity-related ailments in general.

Background Art

High serum triglyceride level, independent of the well-known risk factor of serum cholesterol, has been regarded as an additional risk factor for developing cardiovascular diseases, including coronary heart disease (Austin MA, *Am. J. epidemiol.* 129: 249-59,1989) and atherosclerosis (Patsch JR et al, *Arterioscler. Thromb.* 12: 1336-45, 1992). A number of pharmaceutical developments have been made to restrict the elevation of serum triglyceride levels to prevent such cardiovascular ailments.

More significantly, the excessive intake of lipid with respect to energy expenditure leads to obesity, which is currently being regarded as one of the prime health concerns in the Western World. Obesity is a complex medical disorder with implications for diabetes, high cholesterol, cardiovascular conditions, some forms of cancer, and is a major cause of premature mortality. Dietary restriction and behavioral changes are key to prevent obesity, however it is now becoming evident that the success in preventing or treating obesity can be increased with pharmaco-therapy. Several drugs have been developed to combat obesity, however most of

these are central-nervous system (CNS)-active, and hence have high abuse potential. Therefore, it would be desirable to have a pharmaceutical agent that would not have these dependency complications.

Recently, a group of low molecular weight peptides which were originally obtained and purified from a non-specific enzymatic proteolysate preparation of bovine reticulocyte protein has been shown to inhibit the elevation of serum triglyceride levels (US Patent 5,958,885). The peptides isolated are low molecular weight, i.e. 3-4 residues in length, and are comprised solely of natural amino acids.

Now, retro-inverso technology, in which oligopeptides are synthesised that are similar to naturally occurring oligo-peptides but with mirror image amino acids put in reverse sequence order (Chorev M, Goodman M, TIBTECH 13:438-45, 1995), is a technology that has had limited take-up in recent years. By utilizing non-natural D-amino acids instead of L-amino acids, it can provide an advantage in bioavailability due to inherent resistance against various natural proteases *in vivo* but there is no expectation for its use to effectively mimic or better the biological action of naturally occurring peptides.

Disclosure of the Invention

The present invention provides a pharmaceutical composition or a food composition for administration to a human or an animal comprising a retro-inverso isomer peptide as an active component.

Best mode for carrying out the Invention

In one aspect of the present invention there is provided a pharmaceutical composition for administration to a human or an animal comprising , as an active component, a peptide selected from the group consisting of D-Pro D-Tyr D-Val D-Val, D-Pro D-Tyr D-Val, and D-Leu D-Thr D-Val.

These peptides have unexpectedly been found to have a biological activity to reduce serum triglycerides. Among them, the peptides of D-Pro D-Tyr D-Val and D-Leu D-Thr D-Val substantially exceed the activity of the corresponding natural oligopeptide.

Unlike the natural oligopeptides, the peptides obtained from the retro-inverso chemical synthesis process are also available for functional group modifications, if required. This modification further allows for greater specificity and selectivity, and will also permit to tailor the activities of the peptides to suit the patient based on his/her intake of fat composition.

The peptide may, within the scope of the claimed invention, have minor modifications of a nature that is compatible with biological systems, suitably including phosphorylation, sulphonation or iodination of the D-Tyr and / or D-Thr. The D-Pro may, for example, be hydroxylated. Widely used automatic solid-phase peptide synthetic methods for performing the modification include N-alpha-acetylation or N-alpha-formylation for eliminating the positive charge of the N-terminus. When desiring to eliminate the negative charge on the C-terminus, a C-terminal carboxamide or alcohol ester can be readily generated by adopting standard solid-phase peptide synthetic resins.

For optimal activity, the peptide may be modified at the N or C, and suitably both, terminals of the peptide.

Complete reversal of ionization state can also be straightforwardly performed. The N-terminal NH_2 group is suitably replaced with a COOH group and the C-terminal COOH group is suitably replaced with an NH_2 group. This modification may suitably be undertaken using one of the conventional techniques for this purpose.

A route for this modification involves:

(1) a C-2 substituted malonyl (or malonamyl) residue substitution for the N-terminal retro-inverso peptide residue, and

(2) a *gem*-diamino alkyl residue substitution for the C-terminal retro-inverso peptide residue.

The pharmaceutical composition is suitably provided in a form selected from the group consisting of a tablet, a powder, a granule, a pill and an injectable form. If provided in an injectable form it is suitably selected from the group consisting of a solution, a suspension and an emulsion.

The said injectable form may be administered by intravenous, intramuscular, subcutaneous, intracutaneous and intraperitoneal administration. The pharmaceutical composition suitably comprises from 1 to 100 mg of said peptide.

According to a second aspect of the invention there is suitably provided a food composition for administration to a human or an animal comprising a peptide consisting of D-Pro D-Tyr D-Val D-Val, D-Pro D-Tyr D-Val, and D-Leu D-Thr D-Val as an active component.

The present invention is further illustrated and described by the following examples, which should not be taken to limit the scope of the invention.

Example 1. Chemical Synthesis and Purification of Peptides

Peptides of D-Pro D-Tyr D-Val D-Val, D-Pro D-Tyr D-Val and D-Leu D-Thr D-Val were synthesized on an Applied Biosystems/Perkin-Elmer 432A Synergy Peptide Synthesizer using FastMoc cycles. The synthesis chemistry involves 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) / piperidine activation, and uses dimethylformamide (DMF) / N-methylpyrrolidine (NMP) / dimethylsulfoxide (DMSO) as the coupling solvent. Synergy Fmoc-Amide resin (Applied Biosystems/Perkin-Elmer) or Rink amide methylbenzhydrylamine (MBHA) resin (NOVAbiochem) was used for the solid-phase support. The constituting N-α-9-fluorenylmethoxycarbonyl (Fmoc) protected D-amino acids (N-α-Fmoc-D-proline, N-α-Fmoc-O-t-butyl-D-tyrosine, N-α-Fmoc-D-valine, N-α-Fmoc-D-leucine, N-α-Fmoc-O-t-butyl-D-threonine) were from

NOVABIOCHEM. The peptides were cleaved by adding 1.8 ml of trifluoroacetic acid (TFA) with 0.1 ml of 1,2-ethanedithiol (EDT) and 0.1 ml of thioanisole as scavengers for 1 hour, then precipitated with 15 ml of methyl tert-butyl ether (MTBE) at 4°C and centrifugation at 2000 xg. The MTBE washing was repeated three more times, and the peptides were solubilized with 20% acetic acid. To use as reference compounds, L-Val-L-Val-L-Tyr-L-Pro, L-Val-L-Tyr-L-Pro, and L-Val-L-Thr-L-Leu peptides were prepared using the same methodology.

When necessary, purification of the peptides was performed using preparative reversed-phase HPLC. A Kromasil KR-100-10-C8 (10 mm x 250 mm, C8, 10 μm, 100 A, Akzo Nobel) Column was used, with a linear gradient of 5 to 20% acetonitrile (CH_3CN) in 0.1% TFA over 20 column volumes. The fractionated peak was checked for purity using a Vydac (Registered Trade Mark) 218TP52 RP-HPLC column (2.1 mm x 250 mm, C8, 5 μm, 300 A) with a linear gradient of 1 to 25% CH_3CN in 0.1% TFA. The final purity of each peptide was greater than 97%. MALDI-TOF mass spectrometry analyses using cinnapinic acid as matrix on a Kompact Research MALDI IV instrument (Kratos Analytical) confirmed the identities of the peptides.

Example 2. Oral Administration of Chemically Synthesised Peptides to Determine Inhibition of Triglyceride Level Elevation After Feeding

Olive oil (250 mg) was administered via gastric intubation to male ICR mice (6-week old, body weight: 20 g), which were fasted overnight. The peptides were dissolved in 0.1 ml saline solution and administered orally one hour after lipid administration. For the control group, 0.1 ml of saline solution was administered per mouse. After two hours, blood was collected from the orbital vein under ether anesthesia and the serum was separated by centrifugation (3000 rpm, 30 min, 1°C). Serum triglyceride levels were assayed using commercially available methods

(e.g. INFINITY (Registered Trade Mark) triglycerides reagent; Sigma Chemical Co.). The results were compared with L-Val-L-Val-L-Tyr-L-Pro (Reference Peptide 1), L-Val-L-Tyr-L-Pro (Reference Peptide 2), and L-Val-L-Thr-L-Leu (Reference Peptide 3) and shown in Table 1.

Table 1

	Peptide Dosage (mg / mouse)	Number of animal (n)	Serum Triglyceride (mg / 100 ml)	% Decrease
Distilled Water Only	-	5	92.2 ± 15.7	-
Olive Oil Only	-	24	376.2 ± 23.8	-
SEQ ID No. 1 (D-Pro D-Tyr D-Val D-Val C-NH_2)	1.0	14	313.7 ± 45.9	22.0 %
Ref. Peptide 1 (L-Val L-Val L-Tyr L-Pro)	1.0	7	244.7 ± 25.5	46.3 %
SEQ ID No. 2 (D-Pro D-Tyr D-Val C-NH_2)	1.0	7	231.8 ± 27.6	50.8 %
Ref. Peptide 2 (L-Val L-Tyr L-Pro)	1.0	14	407.7 ± 42.0	0 %
SEQ ID No. 3 (D-Leu D-Thr D-Val C-NH_2)	1.0	7	294.6 ± 33.8	28.7 %
Ref. Peptide 3 (L-Val L-Thr L-Leu)	1.0	14	352.7 ± 35.6	8.3 %

In Table 1, "C-NH_2" means that the C-terminal of a peptide has a carboxamide form.

As shown in Table 1, SEQ ID NO. 2 and SEQ ID NO. 3 display higher activities in lowering elevated serum triglyceride levels than Reference 2 and 3, respectively. SEQ ID NO. 1, although less active than Reference Peptide 1, nevertheless exhibits demonstrable serum

triglyceride lowering activity, its activity being about half of Reference Peptide 1. In general, it can be shown that for the three cases, there exist at least 20 % or greater statistically significant decrease in serum triglyceride levels, and for the cases of tripeptides, the retro-inverso compounds substantially exceed the activity of the corresponding natural oligo-peptides.

As noted previously, for enhanced efficacy the retro-inverso peptides suitably have modified N and C terminals, where the N-terminal of the retro-inverso peptide is converted to replace the NH_2 group with a COOH group and the C-terminal COOH group is replaced with an NH_2 group. This modification may be achieved by carrying out a C-2 substituted malonyl (or malonamyl) residue substitution for the N-terminal retro-inverso peptide residue (Residue R_1 in Fig. 1), and a *gem*-diamino alkyl residue substitution for the C-terminal retro-inverso peptide residue (Residue R_4).

Figure 1 shows the structural differences between the retro-inverso peptide H-$_D$-R_1-$_D$-R_2-$_D$-R_3-$_D$-R_4-OH (Structure 1) and the end-group modified peptide HO-*m*R_1-$_D$-R_2-$_D$-R_3-*g*R_4-H (Structure II, where *m* and *g* denote malonyl and *gem*-diaminoalkyl residues, respectively).

Procedures to accomplish these end group modifications are well-documented, and are reviewed in Fletcher MD, Campbell MM, Chem Rev 1998, 98: 763-795 and Chorev M, Goodman, Acc Chem Res 1993, 26:266-73.

According to the present invention, it becomes possible to prevent hyperlipemia in human and domestic animals upon administration of the peptides found in the sequence listing. Such treatment is now known to have far reaching benefits, including but not restricted to, cardiovascular ailments such as hypertension and arteriosclerosis, and obesity-related complications in general.